

**CHANGES TO THE SPECIFICATION**

Please substitute the following marked up paragraph(s) for the paragraph(s) now appearing in the currently filed specification:

This application is a divisional application of serial no. 09/633,739 filed August 7, 2000, which in turn is a divisional application of serial no. 08/456,108 filed 5/30/95, now issued U.S. Patent No. 6,100,54, which in turn is a divisional application of serial no. 08/145,681 filed 10/28/93, now issued U.S. Patent No. 5,571,691, which in turn is a continuation in part of pending application serial no. 07/967,947, filed 10/27/92, now abandoned, which in turn is a continuation of application serial no. 07/348,270, filed 05/05/89, now abandoned. This application is also a continuation in part of pending application serial no. 07/878,304 filed 04/24/92, now abandoned.

Page 6, line 6-8

Figs. 2A through 2D collectively depict is the cDNA sequence (SEQ. ID No. 1) with deduced amino acids (SEQ. ID No. 2) for the human lactoferrin protein and signal peptide.

Page 6, lines 18-21

Figs. 7A through 7C collectively depicts an RNA analysis of transformant versus control A07.

Figs. 8A through 8C collectively shows the silver stained SDS-acrylimide gel analysis of recombinant LF secretion and purification.

Figs. 9A through 9B collectively illustrates the characterization of recombinant human LF.

Page 6, lines 26-27

Figs. 12A through 12B collectively shows the expression and purification of the glutathione S-transferase/LFN-1 fusion protein.

Page 7, lines 1-4

Figs. 14A through 14C collectively depict~~is~~ the (A) cDNA sequence (SEQ. ID No. 3) with (B) deduced amino acids (SEQ. ID No. 4) for the bovine lactoferrin protein.

Figs. 15A through 15B collectively depict~~is~~ the (A) cDNA sequence (SEQ. ID No. 5) with (B) deduced amino acids (SEQ. ID No. 6) for the porcine lactoferrin protein.

Page 7, lines 9-14

Figs. 18A through 18N collectively depict~~shows~~ restriction enzyme cleavage sites for the human cDNA sequence.

Figs. 19A through 19M collectively depict~~shows~~ restriction enzyme cleavage sites for the bovine cDNA sequence.

Figs. 20A through 20N collectively depict~~shows~~ restriction enzyme cleavage sites for the porcine cDNA sequence.

Page 11, lines 5-14

Figs. 2A through 2D collectively depict~~is~~ the cDNA sequence (SEQ ID No. 1) with the deduced amino acids (SEQ ID No. 2) for the secretion signal peptide and the mature human lactoferrin protein. The numbers on Fig. 2 correspond to the nucleotides starting at the '5 end. There are binding sites for two iron atoms with four amino acids participating in the binding of each iron. The amino acids at positions Asp80, Tyr112, Tyr209, and aHis273 are required for coordination with one iron, and amino acids at positions Asp415, Tyr455, Tyr548, and His617 bind the other. There are two glycosylation sites at positions Asn157 and Asn498. The numbers refer to the deduced amino acid sequences. There are 25 amino acids per line of protein sequence (starting at nucleotide 18).

Page 14, lines 19-29

The invention also comprises partial sequences of the cDNA of SEQ ID No. 1, 3 and 5 and substitution analogs thereof which code for biologically active polypeptides having homology with a portion of lactoferrin, especially those that are not available from enzyme digests of natural lactoferrins, the method of making polypeptides by use and expression of

partial cDNA sequences, and the polypeptide products produced by the methods of this invention. The desired partial sequences can be produced by restriction enzyme cleavage, as for example at the cleavage sites indicated in Figures. 18A through 18N, 19A through 19N and 20A through 20N. The partial sequences may also be synthesized or obtained by a combination of cleavage, ligation and synthesis, or by other methods known to those skilled in the art.

Page 16, lines 13- Page 17, lines 10.

Fig. 4 is a schematic representation of the human lactoferrin protein precipitated after *in vitro* transcription and translation of the human lactoferrin cDNA. The 2140 bp cDNA was from the human prostate cDNA library and included the 5' untranslated region and the rest of the base pairs correlative to the cDNA sequence of Figs. 2A through 2D omitting the last 208 bp at the 3' terminus. The 2140 bp cDNA was ligated to the EcoRI site of the plasmid vector pGEM<sub>4</sub> (commercially available from Promega Biotech., Madison, WI 53711-5305) downstream from the SP<sub>6</sub> promoter. The plasmid construct was linearized at the 3' end of the hLF cDNA using the restriction enzyme Hinc II or Xba I. The linear DNA template was then transcribed *in vitro* using purified SP6 RNA polymerase in the presence of ribonucleotides as described in the manufacturers protocol (Promega Corporation 1988/1989 Catalogue and Applications Guide). The resultant mRNA was translated using 100ng mRNA template and micrococcal nuclease treated rabbit reticulocyte lysate (as described by Promega) in the presence of 75μCi <sup>35</sup>S methionine (800 ci/mmol, Amersham). *In vitro* synthesized lactoferrin was immunoprecipitated by incubating 100μl aliquots of translation reaction with 10μg of rabbit anti-human lactoferrin IgG (Sigma Chemical Company, St. Louis, MO 63178) for 2 hours at 4°C in 50mM Tris, pH7.5/0.15M NaCl/0.05% Tween-20 (1P buffer). The reaction volume was 200μl. Immunoreactive lactoferrin was precipitated after incubation for 1 hour with 50μg of Protein A sepharose (Pharmacia, Upsalla, Sweden). Immunoprecipitation was carried out by centrifugation for 5 minutes at 10,000g and the precipitate was washed 5 times with 4 volumes of 1P buffer. Total translation products and immunoprecipitates were then subjected to electrophoresis in denaturing 7.5% polyacrylamide gels. After fixing in 50% methanol, the gels were incubated in En<sup>3</sup>Hance (NEN, DuPont, Wilmington, DE 19801) for 1 hour and washed with distilled H<sub>2</sub>O. The gel was then dried under vacuum and exposed to Kodak X-OMAR XAR film at -70°C.

Page 20, lines 12-20

With reference to ~~FIGURE~~Fig. 5, *Aspergillus oryzae* expression plasmid, pAhLFG contains 681 bp of 5' flanking sequence of the *A. oryzae* AMY II gene with includes the signal sequence and first codon of mature  $\alpha$ -amylase. The cDNA coding for mature human lactoferrin is subcloned in frame downstream from these sequences allowing recombinant protein production by the addition of starch to the growth medium. The *Aspergillus niger* glucoamylase 3' untranslated region provides the transcription terminator and polyadenylation signals. The plasmid also contains the *Neurospora crassa pyr4* selectable marker and an ampicillin resistance gene.

Page 21, lines 20-26

With reference to ~~FIGURE~~Fig. 6, Southern blot analysis was performed on transformed *Aspergillus oryzae* strains. Genomic DNA from individual transformants and control AO7 were hybridized with a radiolabelled hLF cDNA probe (2.1 kb). The arrow points to a radiolabelled fragment (2.8 kb) generated upon EcoRI digestion of the expression plasmid which is present in all the transformants (#1-9) but is absent in control untransformed AO7. Molecular weights of bacteriophage lambda Hind III fragments are indicated at the left.

Page 22, lines 18-26

With reference to ~~FIGURE~~Figs. 7A through 7C, RNA analysis of transformant versus control AO7 was performed. In Panel A, Northern analysis of RNA (20 $\mu$ g) from control AO7 and transformant #1 where hybridized with radiolabelled human LF cDNA. Human LF mRNA (923 bp) was detected in the transformant #1 but not in the control untransformed AO7. The positions of the 28S and 18S rRNA bands are indicated on the left. In Panel B, Dot blots of RNA (5 and 10  $\mu$ g) from control AO7 and transformant using radiolabelled human LF cDNA probe as illustrated.

Page 24, lines 20-page 25, lines 5

With reference to ~~FIGURE~~Figs. 8A through 8B, panel A illustrates a Silver stained SDS-polyacrylamide gel analysis of recombinant human LF secretion and purification. Lane

1 contains breast milk human LF standard (500 ng). Lanes 2 and 3 contain samples of the growth medium (40 µg) from induced control AO7 and transformant #1 respectively. Lanes 4-8 contain 100 µl aliquots of eluted fractions (#25, 30, 35, 40, and 45 respectively) collected from the CM-sephadex purification of recombinant LF from the growth medium of transformant #1. The position of the molecular weight markers (BioRad Richmond, CA) are indicated on the left. Sizes are given in kilo Daltons. Panel B illustrates a Western immunoblot analysis of duplicate samples as described in panel A using a specific polyclonal antibody directed against human LF with detection with  $^{125}$ I-protein A.

Page 25, lines 18-30

With reference to ~~FIGURE~~ Figs. 9A through 9B, recombinant human LF was characterized. Panel A illustrates the deglycosylation of lactoferrin. Western analysis of glycosylated and deglycosylated lactoferrin using a specific polyclonal antibody was directed against human lactoferrin with detection with  $^{125}$ I-protein A. The first panel contains authentic breast milk human LF (500 ng) untreated (-) and treated (+) with N-glycosidase F. The second panel contains purified recombinant human LF (500 ng) untreated (-) and treated (+) with N-glycosidase F. The size of glycosylated human LF is indicated with the arrow. Panel B illustrates a functional analysis of recombinant lactoferrin with regard to iron-binding capacity. Panel A and B show the  $^{59}\text{Fe}$  filter binding assay of duplicate samples of authentic breast milk human LF and purified recombinant human LF, respectively, at the concentrations indicated. The first lane in both panels contain BSA (5 µg) as a negative control.

Page 26, lines 27-30

With reference to ~~FIGURE~~ Figs. 2A through 2D, the complete cDNA sequence for human lactoferrin protein is depicted. The cDNA coding for lactoferrin is used to create plasmids and transform eukaryotic cells and to produce the lactoferrin protein.

Page 29, lines 1-7

The results of the Western analysis are shown in ~~Figure~~ Fig. 10. An immunoreactive band at the expected size (50 kDa) for the hLF 3' iron-binding domain was evident in the

cellular extract from induced cells and was absent in control uninduced cells (~~Figure-Fig.~~ 10, lanes 1 and 2). The hLF 3' iron-binding domain associates with the cellular homogenate insoluble fraction (~~Figure-Fig.~~ 10, lane 3) and hence required a further solubilization step in a denaturation buffer to prepare the hLF in a soluble form (~~Figure-Fig.~~ 10, lane 4).

Page 29, lines 8-17

Analysis of a coomassie-stained SDS-PAGE gel also showed the presence of a 50 kDa protein in the cellular extract from induced cultures which was absent in control uninduced cultures (~~Figure-Fig.~~ 11, lanes 2 and 3). The recombinant protein was expressed at levels up to 10 mg/l and represented approximately 5% of the total cellular protein. The hLF 3' iron-binding domain did not associate with the soluble homogenate fraction (~~Figure-Fig.~~ 11, lane 4) and hence required a further solubilization step in a denaturation buffer to prepare the hLF in a soluble form (~~Figure-Fig.~~ 11, lane 5). Purification and solubilization of the recombinant hLF 3' iron-binding domain resulted in a 50% yield of recoverable protein and represented the major protein band in this fraction.

Page 31, lines 9-21

Samples from the solubilized extracts and the purification fractions were analyzed by SDS/PAGE followed by silver-staining. The results of this analysis are shown in ~~Figure-Fig.~~ 12A through 12B. A band at the expected size (32 kDa) for the glutathione S-transferase/LFN-1 fusion protein was detected in the solubilized protein extracts from induced JM109 cultures transformed with pGEX-3X/LFN-1 and was absent in uninduced cultures (~~Figure-Fig.~~ 12A, lane 1). The fusion protein was successfully purified to homogeneity over a glutathione sepharose 4B column (~~Figure-Fig.~~ 12B, lanes 1 and 2). Protein concentration determination using the Bradford reagent (BioRad, Richmond, Ca) showed that the glutathione S-transferase/LFNI fusion protein was purified at levels up to 5mg/l. The GST fusion protein has a protease cleavage site for the protease Kex II between GST and the 52 amino acid protein.

Page 32, lines 22-30 and Page 33, lines 1-3

A schematic representation of this expression plasmid is outlined in ~~Figure-Fig.~~ 13. Restriction enzyme digestion of this expression plasmid with Nsi I, followed by repair using

DNA polymerase I allows subcloning of any cDNA of interest in frame with the  $\alpha$ -amylase signal sequence and alanine residue from the start of the mature  $\alpha$ -amylase II gene. 5' and 3' oligonucleotide primers are designed to contain Acc I ends, and used to obtain the full length cDNA encoding for mature porcine and bovine lactoferrin using polymerase chain reaction (PCR) amplification of their known DNA sequence. The PCR fragment thus obtained is digested with Acc I and repaired using the Klenow fragment of DNA polymerase I for in frame subcloning into Nsi I blunt-ended pAG. The plasmids are then be transformed into the *pyrG*- strain of *A. Oryzae* to obtain expression and secretion of these cDNAs as previously prescribed for human lactoferrin.

Page 34, lines 27-30

The results of the western analysis are shown in ~~Figure~~Fig. 16. An immunoreactive band at the expected size (78 kDa) for hLF was evident in the cellular extract from transformed *S. Cerevisiae* cells. Fig. 16, lane 1.

Page 40, lines 14-23

~~Figs. 18A through 18N collectively depict shows~~ the restriction enzyme cleavage sites in the SEQ I.D. No. 1 cDNA for cleavage by various endonucleases. Table 2 lists the alternative codons that code for the 20 common amino acids. DNA sequence substitution analogs that also code for human lactoferrin can be constructed by choosing alternative codons from Table 2 to alter the DNA sequence between a pair of cleavage sites selected from Figs. 18A through 18N. Alternative codons are assembled into a synthetic oligonucleotide by conventional methods and the synthetic oligo is substituted into the endonuclease treated DNA of Sequence ID. No. 1 by the methods described in "Molecular Cloning. A Laboratory Manual", 2d Edition, Cold Spring Harbor Laboratory Press (1989), to produce a substitution analog.